Morphologic and Molecular Aspects of Glioblastomas

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KEYWORDS

• Glioblastoma • GBM • Pathology • Molecular testing • Methylation profiling

KEY POINTS

- The definition of glioblastomas has continually evolved from a reliance on strict morphologic features to a combination of histologic and molecular criteria, as the understanding of the genetic basis of these tumors expands.
- Modern pathologic workup of glioblastomas includes intraoperative evaluations with tissue-sparing techniques, histologic assessment with immunohistochemical markers, and comprehensive molecular characterization aiming at personalized targeting of genetic abnormalities.
- Machine learning analysis of DNA methylation profiles is a breakthrough technology that has bolstered central nervous system tumor classification and discovery and is particularly beneficial for the diagnosis and subtyping of glioblastomas.

INTRODUCTION

Glioblastomas have been frustratingly refractory to significant therapeutic progress over the last century and remain associated with a dismal prognosis.[1] However, the development of technologies that speed up molecular research has paved the way to significant advances in the understanding of the biology of this class of tumors and opened the horizon for the introduction of potential targeted therapies. As the knowledge expands, concepts and definitions need revisions.

It is now accepted that the original descriptions of glioblastoma *multiforme* represent an amalgam of various neoplasms with diverse, even sometimes mutually exclusive, genetic abnormalities and biologic behaviors. The very concept of glioblastoma has evolved through time, starting with the dropping of the "multiforme" qualifier. With the seminal discovery of *IDH1* gene mutations as drivers of prognosis of glioblastomas,[2] a major split in the classification of glioblastomas into 2 major types, IDH-mutant and IDH-wildtype, was introduced in the 2016 edition of the World Health Organization (WHO) classification of tumors of the central nervous system (CNS), for the first time incorporating molecular criteria into the very definition of glioblastomas.[3]

For the next edition of the WHO classification, slated for release in 2021, glioblastomas are poised to be even further defined on a molecular basis.[4] Lower-grade glial neoplasms that show molecular features of glioblastomas have been shown to behave in a similar fashion and will thus be sanctioned to be called as such.[5] The incorporation of artificial-intelligence techniques to classify CNS tumors based on methylome profiling is emerging as a promising technique to assist in diagnosis and research.[6]

This rapidly changing landscape calls for periodic stops to make sense of the current status of the field. In this review, the authors detail morphologic features of glioblastomas, including those of diagnostically significant subtypes, followed by

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Neurosurg Clin N Am 32 (2021) 149–158
https://doi.org/10.1016/j.nec.2021.01.001

commentaries on the immunohistochemical and intraoperative evaluations of these tumors. A summary of the current understanding of the molecular bases and classification of glioblastomas ensues. The final section discusses the use of DNA methylation profiling as a tool to advance research and diagnosis of CNS tumors, in general, and glioblastomas, in particular.

MORPHOLOGIC FEATURES OF GLIOBLASTOMAS

Glioblastomas are hypercellular proliferations of atypical glial cells, which diffusely infiltrate brain parenchyma (Fig. 1A). The neoplastic cells are pleomorphic, but most characteristically have enlarged hyperchromatic nuclei with clumped chromatin and irregular outlines and variable amounts of cytoplasm from which emanate thick and stout fibrillary processes. Normal CNS astrocytes, on the other hand, even when in a reactive state, have in comparison a smaller and regular elongated nucleus with evenly distributed chromatin, in addition to a cytoplasm with fine and long fibrillary processes. Reactive astrocytes also keep a regular distance from each other, whereas glioblastoma cells heap up disorderly. Glial fibrillary acidic protein (GFAP) immunostains exquisitely highlight these differences (Fig. 1B, C).

The variability of individual neoplastic cell morphology is illustrated by the occurrence and occasional predominance of foamy cells,[7] gemistocytic cells (having an abundant eosinophilic cytoplasm), multinucleated giant cells, and cells with metaplastic differentiation.[8] However, the pleomorphism can be less conspicuous in certain tumors, in particular, small cell glioblastomas and others that present with marked oligodendroglial-like features.[9] These neoplasms show a monomorphic proliferation of oval bland nuclei, indistinguishable from anaplastic oligodendrogliomas on morphologic grounds, the differentiation from which rests on molecular testing.

Glioblastomas, as diffuse glial neoplasms, have the capability of widely infiltrating brain parenchyma without effort. There tends to be a higher concentration of neoplastic cells in the center of the tumor, with gradually reducing cellularity toward the periphery. However, neoplastic glial cells cannot easily breach histologic barriers. The intrinsic confinement of the proliferation is the basis for the rarity of metastatic disease-neoplastic cells have trouble penetrating vessels - and for unique and diagnostically helpful phenomena, such as the formation of secondary structures,[10] clusters of neoplastic cells percolating around vessels neurons, and the "edges" of brain parenchyma, that is, subpial and ependymal surfaces (Fig. 1D).

Mitotic activity is invariably brisk and a required criterion for high-grade glial neoplasms but can be remarkably variable depending on the area of the tumor. A pHH3 immunostain is helpful in expediting the identification of mitoses in difficult cases. Ki-67 immunostains likewise show an elevated proliferative rate, ranging from 15% to 40%, which is higher in areas with increased mitotic activity.

Current official 2016 WHO criteria for assigning a *glioblastoma, WHO grade 4* diagnosis to a diffuse glioma still require the presence of either microvascular proliferation or necrosis on histologic assessment.[3] These criteria will not be necessary for assigning a grade 4 in the next edition if specific molecular features are identified (*TERT* or *EGFR* genetic alterations, or the combination of gain of chromosome 7 and loss of chromosome 10).[5]

Microvascular proliferation occurs as a response of endothelial cells to stimulating factors originating from neoplastic cells to produce angiogenesis and is observed as vascular structures with multiple layers, often forming glomeruloid structures, with increased mitotic activity[11] (Fig. 1E). In markedly hypercellular tumors, neoplastic cells can obscure the vascular structures on hematoxylin and eosin (H&E) -stained slides. A GFAP immunostain helps delineate hyperplastic vessels as clearings in a background of intensely staining glioma cells (Fig. 1F). There is regional variation to microvascular proliferation, which is more pronounced at the tumor edges and areas close to necrosis, where ischemic neoplastic cells more profusely release stimulating factors. These regions correspond to regions of contrast ring enhancement on MRI, and thus, the presence of microvascular proliferation within a hypercellular glial neoplasm on a biopsy sent for intraoperative consultation is a reliable surrogate that representative tissue from a high-grade tumor has been sampled.

Necrosis in glioblastomas is characteristically of the palisading type,[12] whereby tumor cells are arranged radially in a picket fence-like distribution around a central area of necrosis (Fig. 1G). However, as per current WHO recommendations, any type of tumor cell necrosis can be used to meet the criteria. On the other hand, care must be exercised in evaluating specimens where necrosis may have been the result of treatment, especially radiotherapy.

The pleomorphism of glioblastomas has over time allowed the identification and further classification of specific subtypes with unique biologic

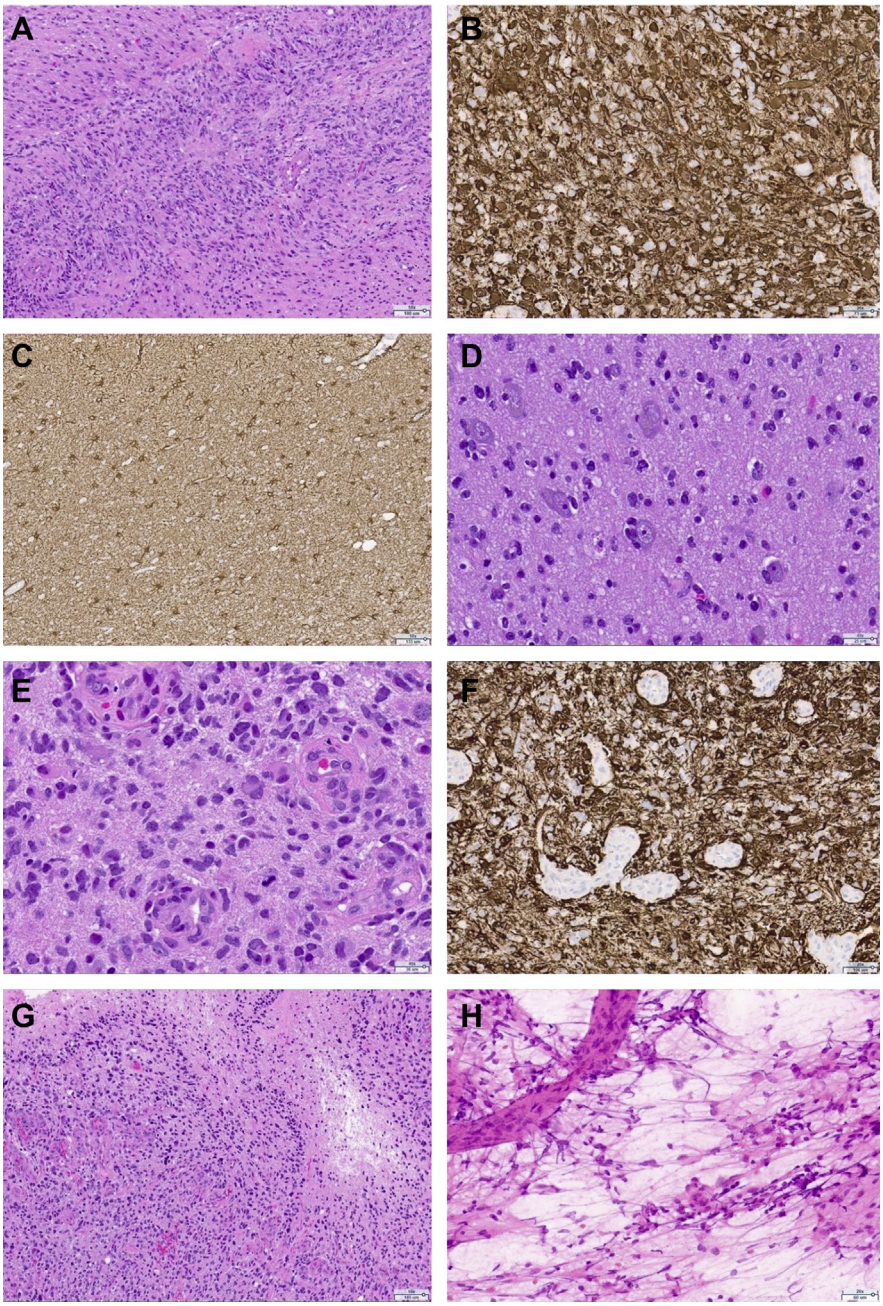

Fig. 1. Morphology of glioblastomas and immunohistochemical stains. (*A*) Hypercellular GBM cells infiltrating brain parenchyma and Virchow-Robin spaces (H&E, original magnification ×100). (*B*) GFAP immunostain highlights cluttered neoplastic GBM cells with strong cytoplasmic and thick fibrillary staining (GFAP, original magnification ×200). (*C*) GFAP immunostain in reactive brain decorates evenly spaced reactive astrocytes (GFAP, original magnification ×100). (*D*) Infiltrating GBM cells forming secondary structures around neurons and blood vessels (H&E, original magnification ×400). (*E*) Glomeruloid microvascular proliferation in a GBM (H&E, original magnification ×400). (*F*) GFAP immunostain in GBM shows negative (unstained) outlines of proliferating endothelial cells in the background of proliferating neoplastic cells (GFAP, original magnification ×200). (*G*) Necrosis in GBM is frequently of the palisading type (H&E, original magnification ×100). (*H*) Smear preparations of GBM specimens show atypical cells with irregular enlarged nuclei and fibrillary processes, associated with proliferating vessels (H&E smear preparation, original magnification ×200).

behaviors and molecular profiles. Giant cell glioblastomas show extremely large, bizarre cells with multiple nuclei, in addition to smaller spindled cells and focal ill-defined rosettes, in a background rich in reticulin (Fig. 2A). They tend to have a more circumscribed architecture, with increased resectability and consequently slightly improved prognosis.[13,14]

Glioblastomas with a primitive neuroectodermal tumor (PNET) component present delimited nests of neoplastic cells differentiated into neuronal, medulloblastoma-like cells within the tumor at large, even showing Homer-Wright rosettes at times, and are associated with abnormalities in *MYCN* (Fig. 2B). Epithelioid glioblastomas (Fig. 2C) are characterized by discohesive rounded epithelioid cells with eccentric nuclei and abundant eosinophilic cytoplasm, sharing molecular features with pleomorphic xanthoastrocytomas (*BRAF* V600E mutation in about half of cases).[15]

Gliosarcomas are tumors with biphasic cells that can either present a glial or spindled sarcomatous morphology, but which have been shown to derive from the same precursor[16] (Fig. 2D). The firm mesenchymal component and the capability to invade the skull are red herrings for a meningioma or metastasis and sometimes pose a radiologic and gross diagnostic challenge. Sarcomatous areas can differentiate into bone, cartilage, and muscle, but the glial component can also take on epithelial features (squamous or adenoid). The sarcomatous component is rich in collagen and reticulin, which can be explored microscopically with a special stain, showing a well-developed intensely staining network around spindle cells.

INTRAOPERATIVE CONSULTATION

The radiologic differential diagnosis of a ring-enhancing CNS lesion, the usual initial presentation for glioblastomas, is broad and includes both neoplasms that require completely different treatment approaches, such as CNS lymphomas and metastases, and numerous nonneoplastic conditions, ranging from infectious diseases to vascular and demyelinating lesions. The intraoperative consultation of a lesional biopsy is thus a critical first step in the workup of a suspected glioblastoma case. Its main purposes are to confirm the diagnosis and to ensure sufficient material has been obtained for the full molecular characterization of the neoplasm, which will guide subsequent therapy.

The ideal biopsy specimen should be representative of the higher-grade area of the tumor, showing unequivocal morphologic features of a high-grade neoplasm (necrosis or microvascular proliferation) and potentially having a high viable tumor cell cellularity, yielding the maximum amount of genetic material for molecular testing.

Various tissue assessment techniques can be used individually or in combination during an intraoperative consultation and include touch and smear preparations and frozen sections. Different services have preferred methods dictated primarily by prior experience. However, for small biopsies of a suspected high-grade glioma, smear preparations offer the advantages of maximal tissue preservation while providing optimal cytologic detail.

Interpretation of smear preparations reliably distinguish gliomas from the main differential diagnoses. Smear preparations of glioblastomas show a predominant population of atypical cells with enlarged elongated nuclei, inconspicuous nucleoli, and a variably sized cytoplasm that characteristically displays fine fibrillary processes (Fig. 1H). The smear background is also finely fibrillary. Processes oriented perpendicular to the direction of the smearing offer stronger evidence that one is not dealing with artifactual disruption of the cytoplasm of other potential tumor cell types. Mitotic figures are occasionally identified, further boosting confidence in a correct diagnosis.

IMMUNOHISTOCHEMICAL EVALUATION

A limited number of immunohistochemical stains are helpful for the initial characterization of diffuse gliomas and include GFAP, IDH1 R132H, ATRX, p53, EGFR, and Ki-67. Olig2 and pHH3 can be occasionally used in certain scenarios. When appropriate, immunostains for the histone H3 K27M mutation and various H3 G34 mutations are also available, as is a *BRAF* V600E stain for epithelioid glioblastomas.

GFAP is expected to diffusely and strongly stain neoplastic cells in glioblastomas, highlighting the thick glial protein content extending into stout abnormal processes that nevertheless recapitulate the astrocytic nature of the cells (see Fig. 1B). The abundant cytoplasm of gemistocytic cells also strongly stains with GFAP. In cytoplasm-poor variants of glioblastoma, and in cases where there is partial loss of GFAP expression in neoplastic cells, such as in gliosarcomas, an immunostain for Olig2 can be helpful in further establishing the glial nature of the neoplasm.[17] GFAP is also helpful in the evaluation of microvascular proliferation when equivocal on H&E, as negative outlines of endothelial cells starkly contrast with strongly staining neoplastic cells.

The IDH1 R132H immunostain stains the abnormal protein product resulting from the specific *IDH1* R132H mutation and is thus negative

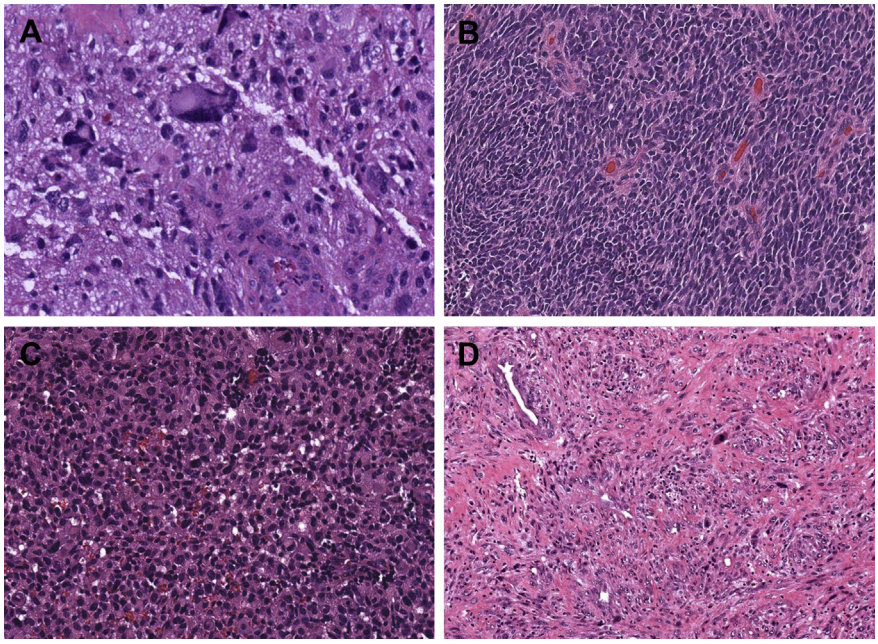

Fig. 2. Morphology of glioblastoma subtypes. (*A*) Giant cell glioblastoma shows bizarre markedly enlarged "monstrocellular" neoplastic cells (H&E, original magnification ×400). (*B*) Glioblastoma with PNET features is characterized by primitive hyperchromatic cells mimicking medulloblastoma (H&E, original magnification ×100). (*C*) Epithelioid glioblastoma is composed of cells with abundant eosinophilic cytoplasm (H&E, original magnification ×100). (*D*) Gliosarcoma shows a prominent spindle cell component admixed with pleomorphic glial cells (H&E, original magnification ×200).

in IDH-mutant gliomas harboring alternative mutations in *IDH1* or *IDH2*. In the initial evaluation of glioblastomas, it helps suggest, coupled with a retained nuclear expression of ATRX, the IDH-wildtype nature of the neoplasm. In patients younger than 55 years or with a prior history of lower-grade glioma, particularly with loss of ATRX expression, the possibility of an IDH-mutant glioma with a noncanonical *IDH1* or *IDH2* mutation must be confirmed with molecular testing. In the right clinical context, immunostains that work with the same principle as IDH1 R132H, to detect a particular mutation, are available for H3 K27M, H3 G34R, and H3 G34V, as is a *BRAF* V600E immunostain when considering an epithelioid glioblastoma.

ATRX retained nuclear positivity is expected in IDH-wildtype glioblastomas, whereas it is lost in IDH-mutant diffuse astrocytomas of any grade, as a result of truncating mutations in ATRX determining loss of protein expression. As for any negative stain, it is crucial to evaluate for the presence of proper internal positive controls in the examined tissue, most frequently by looking for intact nuclear staining in endothelial cells.

The immunostain for p53 shows variable weak positivity in normal brain parenchymal components and in neoplastic cells that do not harbor p53 mutations. In *TP53*-mutated glial neoplasms, 2 abnormal staining patterns are possible. In 1 pattern, a subset of clonally expanded neoplastic cells shows strong intense staining, corresponding to the accumulation of an abnormal protein product, which results from a missense mutation. In the second pattern (null pattern), neoplastic cells have a complete absence of p53 staining, a consequence of the lack of protein expression owing to biallelic truncating (null) mutations. Although diagnostically helpful, especially taken together with staining patterns of other immunostains, the immunohistochemical evaluation of p53 is not completely concordant with the ultimate molecular *TP53* status and therefore does not substitute the latter.

The same can be said of the EGFR immunostain, which can help suggest the presence of *EGFR* amplification when strongly and diffusely positive in the cytoplasm of neoplastic glioblastoma cells, but too frequently shows weak to moderate staining otherwise. The best use of the EGFR immunostain is to evaluate for the presence of individual infiltrating cells in hypocellular samples of recurrent tumors that are known to be EGFR amplified, in a way similar to the use of the IDH1 R132H mutant for recurrences of an IDH-mutant diffuse glioma.

The Ki-67 immunostain is used to establish the proliferative rate, which ranges from 15% to 40% in most glioblastomas and is higher in areas with increased mitotic activity. A pHH3 immunostain decorates mitotic figures and is useful in the occasional equivocal case whereby the morphology of the neoplastic cells or the processing of the sample make mitoses harder to identify.

MOLECULAR FEATURES AND WORKUP

Since the advent of next-generation sequencing (NGS), several custom molecular panels have been developed that cover clinically relevant genetic alterations identified in CNS tumors.[18] These panels include sequencing of DNA to detect frequent point mutations, as well as RNA to capture common fusions.[19] As the knowledge of the genetic landscape of glioblastomas develops further and the technologies become cheaper and more widely available, the panels also evolve in coverage and breadth.

Although the current state of the genetic sequencing technology maturity justifies, from a cost perspective, that panels cover hundreds of genes, at a minimum, an NGS panel for a purported glioblastoma should include evaluation of the following genes: *IDH1*, *IDH2*, *ATRX*, *TP53*, *PTEN*, *TERT*, and *EGFR* (including structural alterations). Assessment of the methylation status of *MGMT* is also considered standard of care but is typically carried out as a separate assay or can be extrapolated from DNA methylation profiling data. In dealing with epithelioid glioblastomas, evaluation for the presence of the *BRAF* V600E mutation can be sought by either molecular testing or immunohistochemistry.

The 2016WHO classification of brain tumors admits glioblastomas into IDH-wildtype and IDH-mutant subtypes based on the mutation status of the genes that codify isocitrate dehydrogenases 1 and 2 (*IDH1* and *IDH2*). IDH-wildtype status is associated with primary glioblastomas, which arise in older patients with a grade 4 morphology at presentation and display a more aggressive biological behavior. Conversely, IDH-mutant glioblastomas were described as a secondary evolution of more indolent lower-grade diffuse and/or anaplastic astrocytomas in younger patients (55 years is the usual threshold) and therefore have a better prognosis. These subtypes are in essence indistinguishable on morphologic grounds, except for subtle but unreliable signs (for example, IDH-mutant tumors have a tendency to show a relative admixture of more well-differentiated astrocytic neoplastic cells). Nevertheless, IDH-wildtype and IDH-mutant glioblastomas represent entirely different tumor classes from a molecular standpoint, and future editions of the WHO classification of brain tumors will reflect that understanding by restricting the glioblastoma designation exclusively to those tumors with an IDH-wildtype status.[4] Going forward, IDH-mutant glioblastomas will likely receive the alternate designation of astrocytoma, IDH-mutant WHO grade 4. Along the same line of reasoning, cases of IDH-wildtype grade 2 and 3 astrocytomas, rarely observed in practice, which correspond biologically either to undersampled or not yet fully morphologically developed IDH-wildtype glioblastomas, will be flat out called glioblastomas, regardless of whether necrosis and microvascular proliferation, signatures of a grade 4 morphology, are present. For the remainder of the discussion in this section, the term glioblastoma will be used interchangeably with IDH-wildtype glioblastoma.

Mutations in *TP53* and *ATRX* are molecular signatures of IDH-mutant gliomas, but are rare in glioblastomas. They correlate inversely with the presence of *TERT* mutations.[20] The *TERT* gene encodes one of the components of the enzyme telomerase, essential for keeping chromosomal telomere lengths from shortening, a mechanism that allows cells to avoid undergoing apoptosis as they age and divide. Noncoding mutations in the *TERT* promoter region lead to an overactive telomerase that overshoots this protective mechanism and permits cells to survive and divide indefinitely. The *TERT* promoter mutations c.−124C > T and c.−146C > T are signature molecular alterations in glioblastomas, present in up to 90% of cases, and can be used as one of the definitional molecular criteria for this class of tumors.[21] Because *TERT* promoter mutations can also be found in other CNS tumors that are part of the differential diagnosis of glioblastomas, judicious integrated molecular and histologic workup must be followed.

The DNA repair enzyme O-6-methylguanine-DNA methyltransferase (MGMT) protects DNA from damage caused by alkylating agents, including temozolomide, a key chemotherapeutic agent in the treatment of glioblastomas. Glioblastomas holding high levels of methylation (silencing) of the *MGMT* gene promoter, which correspond to up to 40% of the total, have not only an improved response to temozolomide but also a better prognosis.[22] *MGMT* methylation is thus considered both a predictive and a prognostic marker and a key component of the molecular workup of glioblastomas.[23] Epidermal growth factor receptor (*EGFR*) amplification is found in 40% of glioblastomas[24] and is one of the molecular criteria that authorizes a grade 4 designation to

morphologically lower-grade gliomas.[5] Of all *EGFR* amplified glioblastomas, about half additionally carry a rearrangement that generates *EGFRvIII*, a variant purported to give rise to a worse prognosis.[24] *EGFR* is not only diagnostically relevant but also represents a potential therapeutic target. Other than EGFR, several proteins involved in the RTK/PI3K/PTEN/AKT/mTOR pathway have been implicated in glioblastomas. *PTEN* mutations are found in up to 40% of cases, *PDGFRA* in 15%, and *NF1* in 20%, whereas occasional cases (<10%) have amplification of *MET* or *PI3K*, or fusions involving *FGFR1* or *FGFR3*.[25] Another pathway frequently altered in glioblastomas is the CDKN2A/CDK4/RB protein pathway, which occurs in up to 80% of tumors, predominantly owing to alterations in CDKN2A and CDK4 (RB1 mutations are rare in glioblastomas but more common in IDH-mutant gliomas). CDKN2A, in particular, is also involved in the p53 pathway. Although the *TP53* gene itself is infrequently mutated in glioblastomas, other proteins of the p53 pathway are implicated in up to 90% of them.[26] These proteins include MDM2, a protein that degrades p53 and is thus tumorigenic when overexpressed, which can occur in more than 50% of cases,[27] and the abovementioned CDKN2A, a tumor suppressor protein that inhibits MDM2.

Genetic alterations identified as a result of running molecular panels should be integrated with clinical, morphologic, and immunohistochemical findings into a final molecular diagnosis, whereby the pathologist exercises the best judgment to reconcile all findings and provides his opinion as how to best interpret the findings.

DNA METHYLATION CLASSIFICATION

Recently, there has been a breakthrough improvement in the characterization of CNS tumors with the development of an artificial intelligence classifier based on DNA methylation profiling.[6] The system has been shown to represent a fast, reliable, and reproducible means to subclassify CNS tumors.

The method is DNA based and works well with low amounts extracted from frozen or formalin-fixed, paraffin-embedded tissue, including old, archived specimens. A streamlined benchwork of a few days can be summarized in a few steps. First, the bisulfite conversion of previously extracted DNA is followed by hybridization of the sample to a standardized microarray chip containing complementary probes to approximately 850,000 genome-wide sites of interest. Then, a scanner reads the chip in a couple of hours and generates a small data file (idat extension) containing normalized methylation levels for all sites of interest. These computer files can be stored indefinitely and occupy little space compared with NGS or imaging data (the average size is a few megabytes).

The classifier was developed using a reference cohort of more than 2000 samples of various types of CNS tumors, including almost all WHO-defined entities. Initially, the methylation data generated from the samples were presented to an artificial intelligence system, which used unsupervised statistical techniques to create clusters of samples with similar methylation profiles, defining methylation "classes." Such computer-defined classes have a dimensionality incomprehensible to the human brain and eye but closely correlate with tumor entities defined by morphologic or molecular features. For example, IDH-wildtype glioblastomas samples have a similar methylation signature and are clustered together by the system (**Fig. 3**A) in a clearly separate class from clusters of IDH-mutant gliomas, which themselves have unique methylation signatures.

Reference samples were then used to train a random forest algorithm to assign a single methylation class to every presented sample methylation profile (idat file), the actual DNA methylation-based tumor classifier. New original samples of methylation profiling, when run on the classifier, receive a class determination together with a confidence score, depending on how close the methylation signature of the sample matches the expected signature of the class. The classifier was validated by running a cohort of an additional 1155 original tumors, including rare and challenging tumors, almost 90% of which were correctly diagnosed, some of which were in conflict with the original pathologic report but substantiated by additional workup.

The DNA methylation classifier can be continuously refined by expanding the reference cohort used to train the algorithm, a process facilitated by the easy exchange of the standardized small idat files. Refinement includes not only subclassification of defined tumor groups but also delineation of new classes. Several tumors to which the classifier fails to assign a methylation class correspond to potentially unrecognized entities that have a unique methylation signature. One such entity is *anaplastic astrocytoma with piloid features*, identified and defined after the methylation analysis of a cohort of pilocytic astrocytomas with high-grade features, which showed that the cohort clustered together with a profile different from existing reference classes.[28] Methylation profiling analyses also speed up identification of clinical

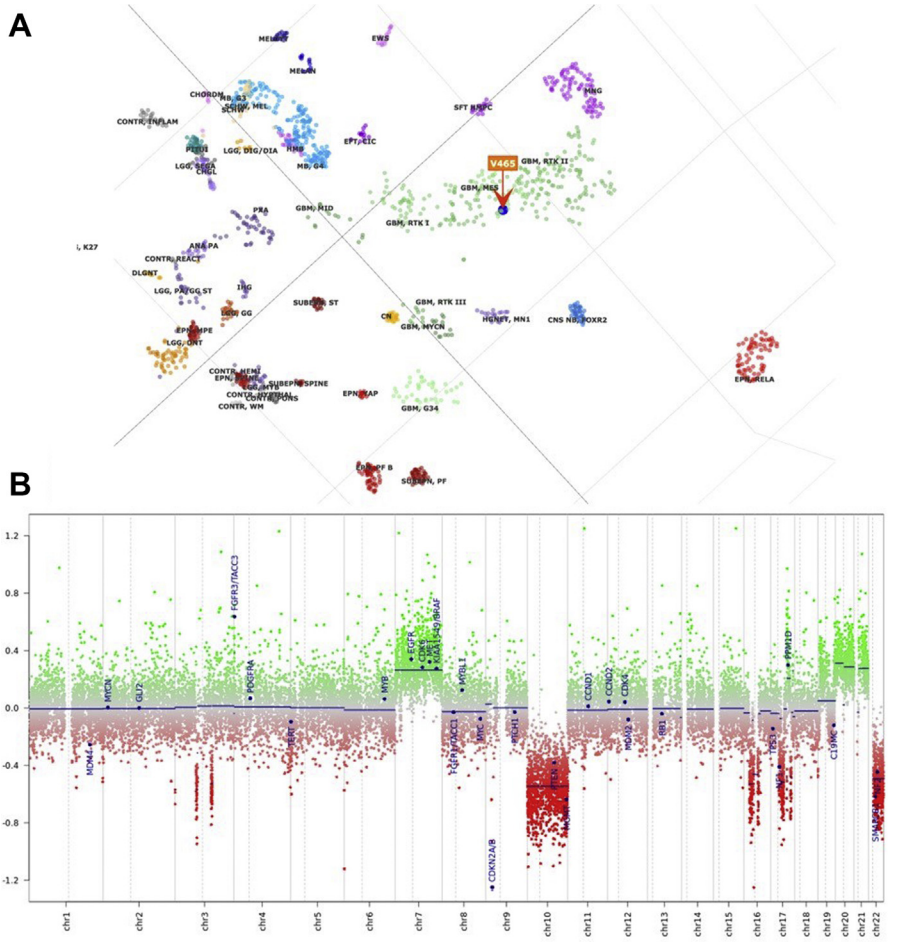

Fig. 3. DNA methylation profiling. (*A*) t-SNE representation of methylation classes defined by the CNS tumor classifier. The interrogated neoplasm (V465) matched to the methylation class "glioblastoma, mesenchymal" (GBM, MES). (*B*) Genome-wide plotting of methylation levels showing detection of copy number variation. This GBM had copy number gains of chromosomes 7, 20, and 21, and copy number losses of chromosomes 10 and 22. There are additionally partial losses in regions of chromosomes 3, 16, and 17.

and molecular subgroups within well-established morphologic entities, with potential prognostic or therapeutic significance.[29] For glioblastomas, there are initiatives to identify methylation subclasses predictive of specific molecular alterations and potential prognostic implications. In particular, glioblastomas harboring FGFR3-TACC3 fusions define a subcluster within the broader glioblastoma, IDH wildtype methylation class (Aldape, 2020).

Methylation signatures have been shown to be specific for cell of origin and to be retained even when neoplastic cells dedifferentiate or metastasize. Methylation data with no match in the CNS tumor classifier can be run on multiple classifiers for further tumor characterization. For example, a "beta-version" of a sarcoma classifier can be used to evaluate skull or dural-based tumors that invade the brain, or a metastatic tumor can be run on specific organ system classifiers for a potential match. The ultimate methylation classifier would be one trained with samples of any human organ system and be capable of solving any metastatic tumor of unknown origin or markedly undifferentiated neoplasms.

A bonus feature of DNA methylation profiling is that the high density of probes allows the graphical genome-wide visualization of methylation levels to serve as a surrogate for copy number variation testing (Fig. 3B). Relative gains or losses of entire or partial chromosomes can be easily identified relative to the baseline average methylation levels, allowing for example, the assessment for the complete loss of chromosome arms 1p and 19q in oligodendrogliomas. More specific "zooming in" of regions of interest can determine the likelihood of

the presence of individual gene amplifications, such as of *EGFR*, or losses of heterozygosity in clinically relevant chromosomal regions, for example, 9p21 (containing *CDKN2A*). Furthermore, queries of specific sites can establish with confidence methylation levels of individual genes. In particular, methylation levels of the MGMT promoter region are easily extracted from methylation profiling data and can avoid the extra cost of the additional molecular test for that purpose.

Most histologically diagnosed glioblastomas are readily diagnosed by histopathology. However, the methylation classifier can be helpful to conform the diagnosis in specific cases and rule out related entities that can mimic glioblastoma on histopathology (for example, anaplastic ependymoma, anaplastic pleomorphic xanthoastrocytoma). Currently, several methylation subclasses of glioblastoma are recognized (GBM_MYCN, GBM_RTK_I, GBM_RTK_II, GBM_RTK_III, GBM_MES, GBM_MID). To date, the clinical relevance of these subclasses remains to be determined. Additional molecular features that are helpful include the copy number changes +7/−10 as well as TERT promoter mutation, both of which are observed in most adult IDH-wildtype glioblastomas. High-level amplification of EGFR, which is not present in all glioblastomas, is helpful when present. An additional advantage of DNA methylation is in the setting of an undersampled IDH-wildtype diffuse glioma, where the requisite histologic hallmarks of glioblastoma are not present. In this setting, the finding of a glioblastoma subtype on the classifier, along with one or more of the genomic alterations noted above, can lead to a diagnosis of "molecular glioblastomas" (or more formally, diffuse astrocytoma with molecular features of glioblastoma, grade 4). Pediatric glioblastomas represent distinct molecular subtypes (they are enriched in RTK_III and GBM-MID), and methylation can be quite helpful in the setting of a pediatric high-grade tumor to conform the diagnosis.

Finally, idat files derived from methylation profiling are relatively small and easy to share, allowing for the consolidation of larger numbers of training sets to develop better classifiers. Archived methylation profiled cases can then be effortlessly revisited.

SUMMARY

In summary, glioblastomas have evolved in concept and definition over time from a neoplasm diagnosed solely on morphologic grounds to an entity with strictly defined molecular features. In the era of personalized cancer genomics and targeted molecular treatments, obtaining a precise molecular diagnosis is of utmost importance.

The optimal workup of a potential case of glioblastoma involves a stepwise approach, which includes early diagnosis at the time of intraoperative consultation while using minimal amount of tissue, obtention of viable tumor in sufficient quantity for molecular studies, workup with classic histologic techniques and immunohistochemistry, and the application of various molecular tests and techniques, which include NGS and DNA methylation profiling.

As the understanding of the biology and molecular aspects of glioblastomas continues to evolve, reevaluations of definitions and classifications of this class of tumors will be periodically necessary.

DISCLOSURE

The authors have nothing to disclose.

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